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Genotoxicity evaluation of two derived products from *Allium* extracts: s-propylmercaptocysteine and s-propyl mercaptoglutathione

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ABSTRACT

Propyl-propane-thiosulfonate (PTSO) is one of the main organosulfur compounds present in *Allium* essential oils with a widely documented biological activity. For this reason, it could be used as a food and feed additive in the agri-food industry. A genotoxicity evaluation of substances and their metabolites present in food is necessary to guarantee the consumer's health following the recommendations of the European Food Safety Authority (EFSA). To evaluate the toxicological profile of derivatives of PTSO, the cytotoxicity, an Ames test, a micronucleus test and the comet assay were performed. Results showed that non-cytotoxic effects were observed in Caco-2 exposed to s-propyl mercaptocysteine (CSSP) and s-propyl mercaptoglutathione (GSSP) (0–450 μM). The mutagenicity index remained in the range of 0.6–1.4 for both compounds, showing no mutagenic effects for the concentrations of 5000–312.5 μg GSSP/plate and 250–15.63 μg CSSP/plate. Moreover, the % binucleated cells with micronuclei were 1.3–2.2 and 1.6–2.7 for GSSP and GSSP, respectively. For comet assays there was no DNA-genotoxic or oxidative damage in a concentration range of 112.5–450 μM. Therefore, we can conclude that these compounds are not genotoxic at the conditions tested. These results support that the presence of CSSP and GSSP in the food/feed is not of concern, although further studies are needed to complete their safety profile.

1. Introduction

Organic sulfur compounds (OSCs) are phytochemical molecules with sulfur atoms in their structure, found naturally in many plants of the genus *Allium* sp., especially in edible species, such as Onion (*Allium cepa*) or Garlic (*Allium sativum*). Although these plants contain flavonoids, polysaccharides, or glucosinolates (Cozzolino et al., 2021), OSCs are their most well-known compounds because of their beneficial properties: antibacterial, antifungal, anti-inflammatory, antidiabetic, etc. (Marimuthu & Ramasamy, 2020; Farhat et al., 2021; Catanzaro et al., 2022). These compounds arise as a consequence of an enzymatic degradation in defense of physical aggressions such as cutting or crushing the plant, and, for this reason, they are considered secondary metabolites (Poojary et al., 2017). Propyl-propane-thiosulfonate ($C_6H_{14}O_2S_2$) (PTSO) is one of these OSCs. Its properties, such as antimicrobial activity or antioxidant capacity, are of great interest for the agri-food industry because of the high demand for new natural additives (Cascajosa-Lira, Prieto, Baños, et al., 2020, 2020b, 2021a, 2021b, 2022a; Mylona et al., 2019). Furthermore, PTSO has shown anti-methanogenic activity in ruminant feed (Martínez-Fernández et al., 2015).

Recently, it has been demonstrated that PTSO added as feed supplement presents beneficial effects such as modulators of the gut microbiota in farm animals (Guillamón et al., 2021), and increase productivity parameters in laying hens and piglets (Abad et al., 2021; Rabelo-Ruiz et al., 2021). In general, the effectiveness of PTSO as feed additive has been verified and may be a good natural alternative to synthetic agents. It has been described that PTSO is capable of reacting with cysteine (CYS) and glutathione (GLU) forming s-propyl mercaptocysteine (CSSP) and s-propyl mercaptoglutathione (GSSP), respectively (Abad et al., 2016; Guillamón, 2018). These thiosulfates conjugates can be occurred in processed foods, in the digestive tract, and through *in vivo* metabolism (Zhang et al., 2010). In fact, they have been recently found in food matrixes in a significant concentration when PTSO is used as additive (Abad et al., 2016).

Moreover, other compounds such as s-allylmercaptocysteine (SAMC) is produced through the combination of chemical and enzymatic

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Abbreviations

A549	lung cancer cell line
B16/BL6	melanoma cell line
BNMN	binucleated cell with micronucleus
BHK21	renal fibroblast cell line
BKA-B	burkitt lymphoma cell line
Caco-2	colorectal cancer cell line
CSSP	s-propylmercaptocysteine
DADS	diallyl disulphide
DAS	diallyl sulphide
DATS	diallyl trisulphide
DPDS	dipropryl disulphide
DPS	dipropyl sulphide
Endo III	endonuclease III
FPG	formamidopyrimidine-DNA glycosylase
FS4	foreskin cell line
GSSP	s-propyl mercaptoglutathione
HepG2	heapatocarcinome cell line
HT-29	intestinal epithelial cell line
MCF7	epithelial cell line
MN	micronucleus
MTS	tetrazolium salt reduction
NDI	nuclear division index
NR	neutral red
L5178Y tk ±	lymphoma cell line
OSC	organosulfur compound
PTS	propyl-propane-thiosulfinate
PTSO	propyl-propane-thiosulfonate
SB	standar break
TP	total protein content
V79	lung cell line

reactions during the aging process garlic extract (Fujii et al., 2018). Important properties have been described in this derivative allyl compound such as inhibition of hepatocarcinogenesis (Xiao et al., 2018) and reduction of cell viability in human colorectal carcinoma cells (Zhang et al., 2014).

In addition, as it occurs with allicin derivatives when reacting with CYS and GLU, both CSSP and GSSP may show interesting immunomodulatory, antitumoral, antioxidant, and anti-inflammatory properties (Imai et al., 2014; Kosuge et al., 2003; Krishnan et al., 2019; Thayumanavan et al., 2017). Furthermore, the mechanism of action of CSSP has been described by several authors. CSSP showed suppression of protein expression (NF-κB, MAPK, and PKC), which in turn declined mRNA expression of NOS and COX-2 as well as reduction ROS, nitric oxide, IL-6, TNF-α, and PGE2, and improved renal functions (Hsu et al., 2004, 2006; Mong & Yin, 2012; Roy et al., 2017; Tsai et al., 2011). Besides, GSSP and CSSP possess less sensory impact than the original compound PTSO, which could be interesting to be used as potential ingredients for food and feed. Despite of these interesting properties, no direct use of these derivatives as a food and/or feed additive is currently described as far as we know. Therefore, since the presence of CYS and GLU is common in food and feed matrices, it is necessary to delve into the aspects related to the toxicity of these compounds, in order to ensure the safe use of PTSO as additive. For this all, it is necessary the characterization of their toxicological profile, considering the consumer's exposure, and including genotoxicity assays, to assure the safety of these substances (EFSA Scientific Committee, 2011). As a first step in the mutagenicity and genotoxicity evaluation of compounds, EFSA requests a battery of two *in vitro* tests to cover the three genetic endpoints (gene mutations, structural and numerical chromosome aberrations) with the minimum number of tests: a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test (EFSA Scientific Committee, 2011; 2016). Because many antioxidant compounds can exhibit pro-oxidant effects, in order to evaluate these effects on DNA bases and to complete this study, the comet assay with and without restriction enzymes was also included.

Although some preliminary studies performed with PTSO showed a possible genotoxic effect *in vitro* (Mellado-García et al., 2015), later *in vivo* studies confirmed that this compound was not genotoxic and could be considered a natural alternative to chemical preservatives used in the food packaging industry (Cascajosa-Lira, Prieto, Baños, et al., 2020, 2021b, 2022; Mellado-García et al., 2016). In contrast, there are no toxicity studies on CSSP and GSSP up to date. Moreover, EFSA Panel on (2012), in its guidance for submission for food additive evaluations, also considers impurities, metabolites, and degradations products of deliberately added substances in food and feed. Also, EFSA (2016) has remarked that "genotoxicity must be evaluated, even if low exposure is expected". Therefore, considering the expected future increased use of OSCs as food/feed additives, as well as the future increased human exposure, to explore the potential muta/genotoxic effects of CSSP and GSSP is worth of research. For all these reasons, more exhaustive studies including decomposition products must be carried out in order to guarantee a safe use of OSCs in the food industry.

Thus, the aim of this study was to evaluate the toxicological profile of CSSP and GSSP to study for the first time their potential cytotoxic effects, as well as their potential *in vitro* mutagenicity/genotoxicity, using the following battery of genotoxicity tests: (1) the bacterial reverse-mutation assay in *Salmonella typhimurium* (OECD 471, 2020); (2) preliminary cytotoxicity assays carried out on the cell lines L5178Y Tk ± and Caco-2; (3) the micronucleus test (OECD 487, 2016) (MN); (4) the standard and enzyme-modified comet assay with Endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (FPG) enzymes in order to determinate the possible oxidation of DNA.

2. Materials and methods

2.1. Synthesis of CSSG and GSSG

The cysteine and glutathione derivatives (CSSP and GSSP), were synthesized by DMC research center following the procedure described by Zhang et al. (2010). Once synthesized, they were analyzed by Ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) to confirm their purity following the procedure described by Abad et al. (2016).

2.2. In vitro bacterial reverse mutation test (Ames test)

The Ames test was performed following OECD Guideline 471 (2020) and Medrano-Padial et al., 2021. Thus, the five recommended strains of *S. typhimurium* were grown under appropriate conditions. Distilled sterile water (negative control), DMSO (solvent control), and the corresponding positive controls for each strain were included. The positive control for TA97A was 9-aminoacridine (50 μg/plate), for TA98 was 2-Nitrofluorene (0.1 μg/plate), for TA100 and TA1535 was Sodium Azide (1.5 μg/plate), and for TA102 was Mitomycin C (2.5 μg/plate).

2.3. Preliminary cytotoxicity assays

For MN test, a previous trypan blue exclusion assay was performed in L5178Y Tk ±cells for 4 and 24 h. Before the comet assay, the basal cytotoxicity endpoints total protein (TP) and tetrazolium salt reduction (MTS) in Caco-2 cells for 24 and 48 h were evaluated. Cell density was determined with an automated cell counter (Invitrogen®, Thermofisher,

MA, USA).The MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) tetrazolium compound (Promega Biotech Ibérica, Madrid, Spain) added to the medium is bioreduced by cells into a colored formazan compound soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 h of incubation in the dark (Baltrop et al., 1991). Total protein assay was performed according to the protocol of Bradford (Bradford, 1976) using Coomassie Brilliant Blue G-250 (BioRad, Madrid, Spain).

2.4. In vitro micronucleus test (MN)

This assay was performed using L5178Y Tk ± cells according to the OECD guideline 487 (2016) and Maisanaba et al. (2015); moreover, our laboratory has an extensive historical database of positive and negative controls to validate our methodology in this cell line. Concentrations were selected based on previous cytotoxicity assays.

2.5. In vitro standard and enzyme-modified comet assay

The comet assays were performed to detect DNA strand breaks and oxidative damage in Caco-2 cells. These assays have been performed in triplicate in which at least 150 cells have been quantified for each assay following the protocol of Collins et al. (1997) with modifications (Llana-Ruiz-cabello et al., 2014; Medrano-Padial et al., 2021). Gastrointestinal cells are the first to be in contact with the substances present in food or feed. For this reason, this model may be the most susceptible to genotoxic or oxidative damage. In this case, Caco-2 cells were chosen as *in vitro* intestinal alternative to understand the complex cellular processes, such as toxicity in a simplified format. For this reason, this model has been extensively used by academia and industry, including our laboratory, for over 30 years (Medrano-Padial et al., 2021). The enzymes Endo III and FPG were used to detect oxidized pyrimidines and oxidized purine (8-oxoGua, ring-opened purines or formamidopyrimidines), respectively.

2.6. Statistical analysis

The analyses were performed using Graph-Pad Prisma 9 version 9.0.0 software.

The Ames test results were statistically analyzed using analysis of variance ordinary one-way (ANOVA) followed by Dunnett's multiple comparison tests. For MN test, a Kruskal-Wallis (non-parametric) test followed by Dunn's multiple comparisons test was performed. Finally, for the comet assay, significant differences about % DNA in tails were calculated by analysis of variance ordinary one-way (ANOVA) followed by Dunnett's multiple comparison test.

3. Results

3.1. Ames test

Insolubility and cytotoxicity tests were necessary to determine the maximum concentrations of the products to be evaluated in the main test, as established by OECD Guideline 471 (2020). Results showed that both products were soluble in DMSO, and GSSP was more soluble than CSSP. GSSP was soluble and non-cytotoxic at the maximum concentration recommended by the OECD (5000 μg/plate), so the selected range was from 31.25 μg/plate to 5000 μg/plate of GSSP. On the other hand, CSSP was insoluble from 250 μg/plate and this concentration was selected as the maximum concentration of the test range (15.63 μg/plate to 250 μg/plate of CSSP). In this case, no cytotoxicity or scoring interference was observed.

In addition, none of the compounds tested showed antibacterial activity against the *S. typhimurium* strains used in the test (TA1535, TA102, TA100, TA98 and TA97A). For any of the strains tested, no significant increases were observed in the number of reverting colonies at the concentrations tested for each substance, GSSP (5000–312.5 μg/plate) and CSSP (250–15.63 μg/plate) (see Table 1). In addition, the MI of tested compounds showed values of 0.7–1.4 for GSSP and 0.6-1-2 for CSSP, always being values less than 2. The positive controls significantly increased ($p < 0.001$) revertant colonies and induced a MI > 2 in all cases, which confirmed the validity and sensitivity of the present work. Moreover, solvent control (DMSO) did not produce any significant differences in comparison to negative controls.

3.2. Cytotoxicity assays

After 4 and 24 h of exposure to both compounds, no significant changes were observed in the trypan blue exclusion assay of L5178Y Tk ±for GSSP (16.25–260 μM) and CSSP (31.25–500 μM) (Fig. 1A and B). Similarly, no significant changes were observed in MTS and TP values of Caco-2 cells exposed to CSSP or GSSP in a concentrations range of 50–450 μM after 24 and 48 h of exposure compared to their respective control group values (Fig. 1C, D, E, F).

3.3. In vitro micronucleus test (MN)

Table 2 shows the results of the frequency of binucleated cells with micronuclei (BNMN %) and the nuclear division index (NDI) in L5178Y Tk ± exposed to GSSP or CSSP in absence of S9 metabolic activation system at two experimental exposure periods (4 and 24 h). In a short period of exposure, the values of BNMN% were 1.3–1.5 range and 1.6–2.6 range, respectively. Moreover, no significant changes neither in BNMN% nor in NDI were observed in comparison to the negative control for any of the products tested. Similar results were obtained for both compounds after longer exposure periods (24 h) (range of 1.8–2.2 for GSSP and 2.0–2.7 for CSSP). In addition, no dose-dependent relationship was observed in the BNMN% values in any of the tests carried out.

Positive controls for clastogens (mitomycin C) and aneugens (colchicine) showed a significant increase in the frequency of BNMN% ($p < 0.001$). Moreover, the NDI values were similar to those of the negative control in all of the experimental conditions assayed.

3.4. In vitro standard and enzyme-modified comet assays

To study the potential direct genotoxic effects on DNA of GSSP and CSSP, the standard comet assay was carried out. Results showed that GSSP and CSSP did not produce genotoxic effects (DNA strand breaks) in Caco-2 cell line at the concentrations tested (450–112.5 μM) after 24 or 48 h of exposure compared to the control group (Fig. 2). In addition, significant differences with respect to the positive control groups were observed in all CSSP and GSSP concentration assays.

In order to evaluate the potential indirect genotoxic effects such as oxidative DNA damage, the FPG and Endo III enzyme-modified versions were included. No significant differences were detected after 24 or 48 h exposure to any of these products in comparison to the negative control in Caco-2 cells analyzed with FPG or Endo III post-exposure (see Figs. 3 and 4, respectively).

Likewise, significant decreased was observed in all concentration assayed compared to positive control groups. In both modified comet assays, a significant increase in DNA oxidation strand breaks was evidenced in positive controls (Ro19–8022 or H_2O_2) indicating the suitability of the assays. The DNA fragments forming the comet tail can be clearly observed in the image in Fig. 5B, compared to the absence of the comet tail in image 5A.

Moreover, the negative and positive controls of both, the standard assay and the enzyme-modified assay are within the ranges established in the historical database for comet assay of our laboratory.

4. Discussion

The numerous beneficial effects of PTSO and the demand for natural

Table 1
Results of the Ames test without S9 exposed to GSSP and CSSP in three independent experiments by triplicate. Milli Q water was used as negative control (100 μl) and DMSO (10 μl) as a solvent for positive controls. Data are given as mean ± SD revertants/plate. Positive controls for TA97A: 9-aminoacridine (50 μg/plate), TA98: 2-nitrofluorene (0.1 μg/plate), TA100 and TA1535: Azide Na (1.5 μg/plate) and TA102: mitomycin C (2.5 μg/plate). Ordinary one-way ANOVA Dunnett's multiple comparison test. Statistical differences: ***when $p < 0.001$ in comparison to negative controls.

		TA97		TA98		TA100		TA102		TA1535	
		Colonies	MI	Colonies	MI	Colonies	MI	colonies	MI	Colonies	MI
GSSP	Negative control	315 ± 14	–	16 ± 4	–	107 ± 16	–	283 ± 37	–	11 ± 2	–
	Solvent Control (DMSO)	254 ± 26	0.8	11 ± 1	0.7	83 ± 29	0.8	298 ± 16	1.1	12 ± 1	1.1
	5000 μg/plate	265 ± 18	0.8	18 ± 3	1.1	80 ± 13	0.8	317 ± 30	1.1	14 ± 2	1.3
	2500 μg/plate	311 ± 20	1.0	16 ± 3	1.0	79 ± 12	0.7	314 ± 10	1.1	13 ± 3	1.2
	1250 μg/plate	257 ± 20	0.8	18 ± 2	1.2	87 ± 7	0.8	312 ± 51	1.1	13 ± 3	1.2
	625 μg/plate	261 ± 25	0.8	18 ± 4	1.2	87 ± 4	0.8	277 ± 13	1.0	15 ± 2	1.4
	312.5 μg/plate	308 ± 18	1.0	17 ± 3	1.1	81 ± 2	0.8	320 ± 30	1.1	13 ± 3	1.2
	Positive controls	708 ± 167***	2.2	930 ± 61***	59.4	615 ± 82***	5.8	571 ± 61***	2.0	468 ± 99***	42.5
CSSP	Negative control	107 ± 29	–	29 ± 3	–	91 ± 2	–	185 ± 13	–	15 ± 6	–
	Solvent Control (DMSO)	108 ± 17	1.0	20 ± 3	0.7	97 ± 24	1.1	135 ± 21	0.7	12 ± 2	0.8
	250 μg/plate	70 ± 7	0.6	31 ± 5	1.1	86 ± 7	1.0	220 ± 20	1.2	13 ± 1	0.8
	125 μg/plate	95 ± 7	0.9	25 ± 3	0.9	84 ± 7	0.9	203 ± 6	1.1	17 ± 4	1.1
	62,5 μg/plate	117 ± 1	1.1	27 ± 2	0.9	80 ± 4	0.9	203 ± 9	1.1	16 ± 2	1.0
	31,25 μg/plate	96 ± 20	0.9	25 ± 2	0.9	88 ± 9	1.0	205 ± 30	1.1	17 ± 2	1.1
	15,63 μg/plate	97 ± 3	0.9	27 ± 2	0.9	89 ± 9	1.0	160 ± 24	0.9	16 ± 2	1.1
	Positive controls	977 ± 50***	9.1	1068 ± 118***	37.3	507 ± 24***	5.6	1060 ± 53***	5.7	540 ± 53***	35.2

additives by consumers have led to an increase in the use of these OSCs in the agri-food industry. In general, the OSCs are reactive and thermally unstable compounds, so their content tends to be reduced over time (Putnik et al., 2019). Reactions between thiosulfonates and cysteine/glutathione are spontaneous and quicker than the reactions with other amino acids (Hsu et al., 2004). Despite of these derivatives compounds have been recently found in food matrixes in a significant concentration (Abad et al., 2016), there is no published information about these cysteine and glutathione conjugate compounds in relation to their safety. Taking into account that EFSA recommends considering metabolites and degradation products for the evaluation of food additives (EFSA Panel on, 2012), the cytotoxicity and genotoxicity evaluation of CSSP and GSSP present a great interest for a safe application of PTSO in the agri-food industry. To our knowledge, this is the first work focused on evaluating the toxic potential of these compounds.

Although the Ames test is one of the assays recommended by the EFSA for food safety assessment, there are very few mutagenicity studies of OSCs. In the present work, no significant changes were observed in the number of reverting colonies at any concentration tested of both derived products. Therefore, GSSP and CSSP showed no mutagenic potential under these experimental conditions. Similarly, PTSO (1–20 μM) and PTS (8.75–280 μM) did not produce mutagenic effects by the Ames test (Mellado-García et al., 2015; Putnik et al., 2019). Moreover, Dipropyl disulfide (DPDS), Dipropyl sulfide (DPS), and their mixture did not show any mutagenic effects in a concentration range of 0.1–200 μM with or without S9 (Llana-Ruiz-Cabello, Maisanaba, et al., 2015). These authors assayed the same strains (T102, TA100, TA98 and TA97A) than the present study with the exception of TA1535 strain. Guyonnet et al. (2000) administered 1 mmol/kg DAS, DADS, DPS or DPDS orally for 4 consecutive days to rats and measured the mutagenic activation capacity of hepatic S9 and microsome samples of these compounds. DADS showed antimutagenic effects against dimethylnitrosamine, N-nitrosopiperidine and benzoapyrene mutagenicity by Ames test using strains TA98 and TA100 (Guyonnet et al., 2000). In contrast, DAS, DPS and DPDS increased the mutagenic effect of cyclophosphamide, N-nitrosopiperidine, 5-b-pyridine and benzoapyrene under the same laboratory conditions (Guyonnet et al., 2000). Likewise, polymer film of *Allium cepa* L. (at 75–100 μl/plate) induced a significant increase in the number of revertant colonies in relation to the respective control groups by the Ames test in absence of S9 (TA98 and TA102 strain) and in presence of S9 (TA100 strain) (Rodrigues-Barreto et al., 2019).

Cytotoxicity endpoints showed that the Caco-2 cell viability was not altered by either of the two compounds (GSSP and CSSP) in a concentration range of 0–450 μM after exposure times (24 h and 48 h) for any of the biomarkers used (MTS and TP). Similarly, in L5178Y Tk ±cells, no significant changes in the viability were observed when the cells are exposed to 0–500 μM for CSSP or 0–260 μM for GSSP after 4 and 24 h of exposure. In relation to the parent compound, similar mean effective concentration (EC_{50}) values for the neutral red (NR), MTS and TP assays for both cell lines (between 350.9 and 388.3 for Caco-2 and 368.1–415.14 μM for HepG2) were obtained for PTSO (Llana-Ruiz-Cabello, Gutiérrez-Praena, et al., 2015). In this sense, CSSP and GSSP turned out to be less cytotoxic than their parent compound PTSO, so their transformation into these derivatives could be a detoxification process. On the contrary, another potential derivative of PTSO, Propyl propane thiosulfinate (PTS) produced greater cytotoxic results in Caco-2 cells exposed for 24 and 48 h, with EC_{50} values of 187–340 μM for these same assays (MTS, TP and NR) (Mellado-García et al., 2017). Thus, the spontaneous reactions produced between PTSO and cysteine/glutathione to form mixed-disulfide conjugates (CSSP/GSSP) occurring in the matrix of animal feed, favors the safety of the products by reducing their cytotoxicity.

With respect to other OSCs, and in agreement to our results, Llana-Ruíz-Cabello et al. (2015) described that other disulfides, such as DPDS, DPS or DPDS/DPS mixture (0–200 μM), showed no significant changes in comparison to negative control in Caco-2 cells by MTS bioassay for the same periods of time (24 and 48 h). By contrast, these authors reported a significant increase of TP at 200 μM of DPDS after 24 and 48 h of exposure and at DPDS/DPS mix (200 μM) only after 24 h. Cytotoxicity has been tested mainly by the MTT test using different cellular models (B16/BL6, MCF-7, BJA-B, BHK21, FS4, HT-29, A549 etc.) being the OSCs investigated potential precursors of GSSP and CSSP: DADS, DATS, DAS, Allicin and Ajoene. Nevertheless, the main aim of these studies was not to assess the toxic potential of these compounds, but to evaluate their antiproliferative effects to justify their potential as chemoprotectants against carcinogenesis. In general, the EC_{50} values obtained were lower than the highest concentration of CSSP and GSSP tested in the present work (450 μM), which were not cytotoxic. Although the vast majority of organosulfur compounds of the genus *Allium* exhibit a strong cytotoxic effect on cancer cell lines (Cascajosa-Lira, Andreo--Martínez, et al., 2022; Xiao et al., 2018; Zhang et al., 2014), in this study, CSSP and GSSP do not show any cytotoxic effect even at high concentrations.

In relation to BNMN percentage and NDI, the L5178Y Tk ±cells exposed to GSSP or CSSP showed no significant changes compared to the control group for any exposure period (4 and 24 h). In agreement with

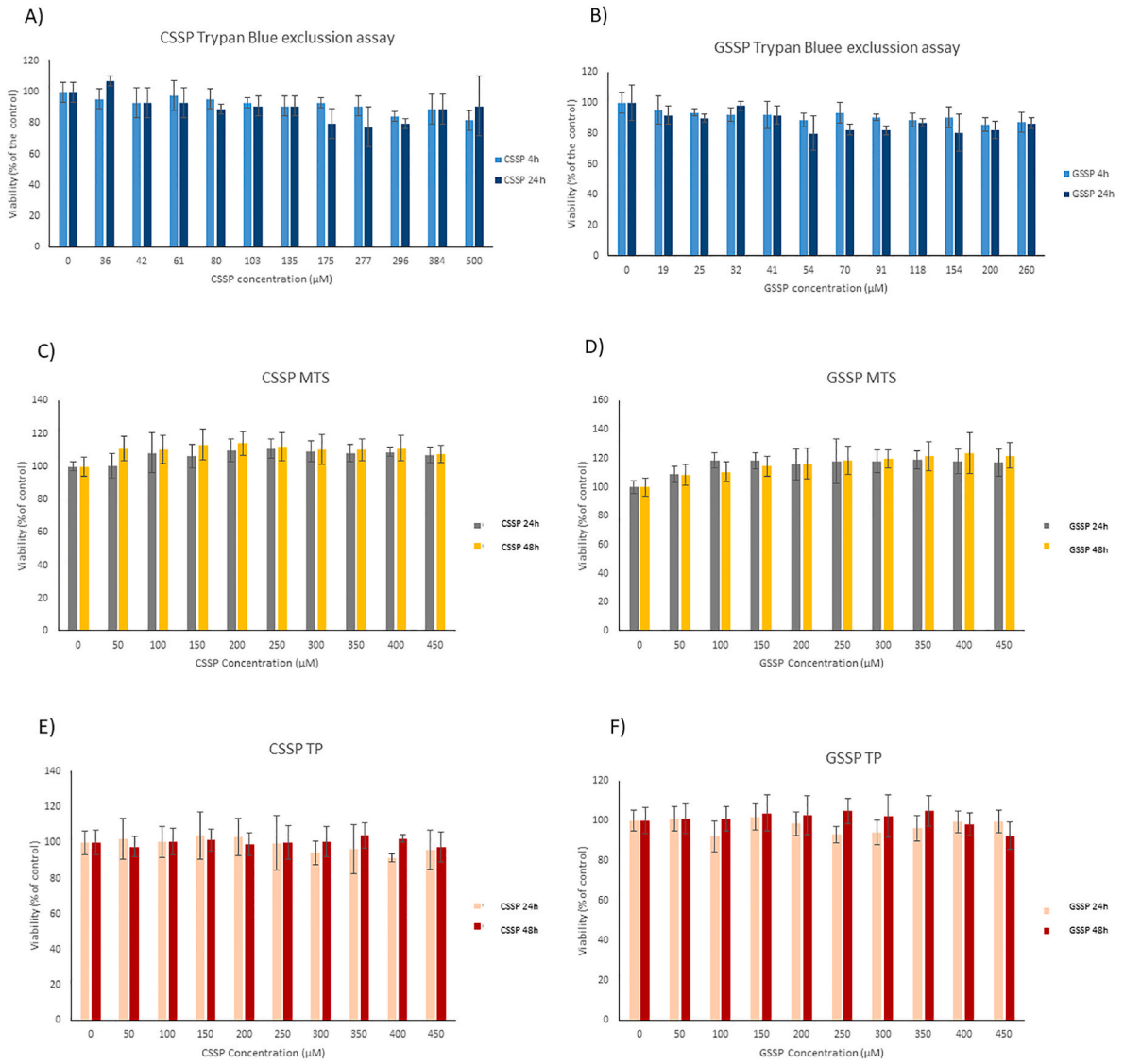

Fig. 1. Cell viability of CSSP A) trypan blue of L5178Y Tk ±for 4 h and 24 h, C) in reduction of tetrazolium salt (MTS) and E) in total protein content (TP) of Caco-2 cells exposed for 24 h and 48 h. Cell viability of GSSP B) trypan blue in L5178Y Tk ±for 4 h and 24 h, D) in reduction of tetrazolium salt (MTS) and F) in total protein content (TP) of Caco-2 cells ex-posed for 24 h and 48 h. All values are expressed as mean ± SD. No significant differences were observed between the exposed groups and their respective controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

our results, PTSO did not produce a significant increase in BNMN L5178Y Tk ±cells exposed to a similar concentration range of 5–40 μM under the same laboratory conditions. In our case, even higher concentrations (up to 260 for CSSP and 500 μM for GSSP) were tested and no genotoxicity was detected. However, in the case of their precursor, a significant increase in BNMN% was shown for the highest concentrations tested in the presence of S9 by these same authors *in vitro* (Mellado-García et al., 2015), although *in vivo* PTSO was not genotoxic (Mellado-Garcia et al., 2016). Therefore, the genotoxic effect could be due to other compounds generated through the metabolic fraction used in these assays. In this sense, PTS, derivative from metabolic reduction of PTSO induced a significant increase in BNMN cells in comparison to negative control at the maximum concentration tested both with and without S9 in this same cell line. More recently, Rodrigues-Barreto et al. (2019) showed non-mutagenic results of an *Allium cepa* polymer film by the MN test carried out on HepG2 cells.

In addition, for both studied derived compounds: GSSP and CSSP, negative results were also found in the present work in the standard comet assay in Caco-2 cells. Moreover, we also demonstrated that these derivates did not induce DNA-oxidative damage (by using the Endo-III and FPG-modified comet assay) in the range of the assayed concentrations (112.5–450 μM). These results agree with those obtained in the comet assay (both standard and modified, 24 y 48 h) by Mellado-García et al. (2016) in Caco-2 cells exposed to PTSO in a lower concentration range than the present study (0–50 μM). On the other hand, Sielicka-Dudzin et al. (2012) showed a significant increase in other allium compound, DATS, which induced DNA damage in PC-3 cells exposed to 40 μM confirming the increase in DNA tail content reported by Borkowska et al. (2012) in the same assay. Similarly, the analogue, DAS, induced concentration-dependent apoptosis in HeLa cells exposed to 0–100 μM for 24 h assessed by the comet test (Wu et al., 2011). Furthermore, no oxidative damage on DNA was also demonstrated for both compounds in the present work (enzyme-modified comet assay). GSSP and CSSP have previously shown antioxidant properties (Zhang et al., 2010). Although at high concentrations these compounds could induce oxidative stress in a similar way that other natural compounds such as N-Acetylcysteine (Puerto et al., 2009), in this case, none of the two investigated compounds exerted prooxidant effects on DNA.

In this context, there are very few studies focused on evaluating the genotoxicity of cysteine and glutathione conjugated compounds.

Table 2
Percentage of the frequency of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured L5178-Y ± cells treated with GSSP and CSSP. The results are expressed as mean ± SD. Clastogen and aneugen positive controls were mitomycin C (0.186 μM) and colchicine (0.031 μM), respectively. The significance levels observed are ***p < 0.001 or **p < 0.01 in comparison to negative control group values.

		BNM (%) ± SD	NDI ± SD	BNM (%) ± SD	NDI ± SD
Exposure time		**4 h**		**24 h**	
Negative control	–	1.7 ± 0.4	1.7 ± 0.3	2.1 ± 0.1	1.8 ± 0.1
Positive control	Mitomycin C 0.186 μM	7.3 ± 0.1 **	1.8 ± 0.1	6.2 ± 1.1***	1.9 ± 0.0
	Colchicine 0.031 μM	7.0 ± 1.1 **	1.7 ± 0.0	6.7 ± 0.4***	1.8 ± 0.1
GSSP	260 μM	1.3 ± 0.1	1.8 ± 0.1	2.0 ± 0.3	1.7 ± 0.1
	130 μM	1.4 ± 0.3	1.8 ± 0.1	1.8 ± 0.3	1.7 ± 0.0
	65 μM	1.3 ± 0.4	1.6 ± 0.1	2.2 ± 0.6	1.7 ± 0.1
	32.50 μM	1.9 ± 0.7	1.7 ± 0.1	2.1 ± 0.1	1.8 ± 0.0
	16.25 μM	1.5 ± 0.4	1.7 ± 0.2	2.0 ± 0.6	1.7 ± .00
CSSP	500 μM	1.8 ± 0.3	1.6 ± 0.1	2.7 ± 0.1	1.8 ± 0.2
	250 μM	2.1 ± 0.1	1.7 ± 0.0	2.4 ± 0.0	1.9 ± 0.0
	125 μM	2.3 ± 0.4	1.7 ± 0.0	2.4 ± 0.3	1.8 ± 0.1
	62.50 μM	1.6 ± 0.6	1.7 ± 0.2	2.0 ± 0.3	1.8 ± 0.0
	31.25 μM	2.0 ± 0.3	1.8 ± 0.1	2.6 ± 0.3	1.8 ± 0.1

Although these conjugation reactions occur generally as a detoxification process, genotoxic results produced by cysteine and glutathione conjugated to other substances have been described. Thus, Zhong et al. (2001) showed that methylenedi-p-phenyl diisocyanate conjugates (BisGS–MDI and BisCYS–MDI) induced a significant increase of MN in V79 cells exposed to 62.5–500 μg/mL during 24 h. Moreover, the cysteine and glutathione conjugates of haloalkenes (1,1,2-trichloro-3,3,3-trifluoro-1-propene and trichlorofluoromethane) have showed concentration-dependent (0–800 nmol/plate) mutagenic effects in TA100 and TA98 *S. typhimurium* strains by the Ames test. It has also been described that glutathione and cysteine methyleugenol conjugates could be involved in the carcinogenicity, cytotoxicity, and genotoxicity of methyleugenol (Yao et al., 2016). In our case, by contrast, this kind of conjugations suggest that the biotransformation of PTSO in these compounds might result in a detoxification process of OSCs.

Therefore, in the present study, after carrying out a complete battery of *in vitro* tests, including the assays required by the EFSA, CSSP and GSSP compounds are shown to be non-genotoxic, so their presence in food/feed products is not of concern in relation to this toxicological endpoint. All these results are needed to understand the potential mechanisms of toxicity of these substances and for a correct risk assessment.

5. Conclusion

The compounds CSSP and GSSP derived from *Allium* did not present mutagenic effects using the Ames test in 5 strains of *Salmonella*. Moreover, L5178Y Tk ±cells showed no genotoxic effect in MN assay for either of the compounds. The derivative products confirmed non-genotoxic effects on Caco-2 cells in the standard comet assay neither oxidative DNA damage in the enzyme-modified comet assay. Consequently, CSSP and GSSP present a good profile for use as a food and/or feed additive due to their biological activities, mainly antioxidant properties. However, other different studies such repeated dose 90-day oral toxicity study in rodents, study on ADME, studies on reproduction and developmental toxicity could be adequate to complete the safety profile of these compounds before their use as active food or feed additive.

CRediT authorship contribution statement

Antonio Cascajosa-Lira: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft; Writing - review & editing; **Concepción Medrano-Padial**: Data curation; Formal analysis; Methodology; Roles/Writing - original draft; **Ana Isabel Prieto**: Data curation; Formal analysis; Investigation; Roles/Writing - original draft; Writing - review & editing; **Alberto Baños**: Conceptualization; Roles/Writing - original draft; Writing - review & editing; **José M. de la Torre**: Conceptualization; Roles/Writing - original draft; Writing - review & editing; **Angeles Jos**: Conceptualization; Data curation; Formal analysis; Investigation; Supervision; Roles/Writing - original draft; Writing - review & editing; **Ana M. Cameán**: Conceptualization; Data curation; Formal analysis; Funding acquisition;

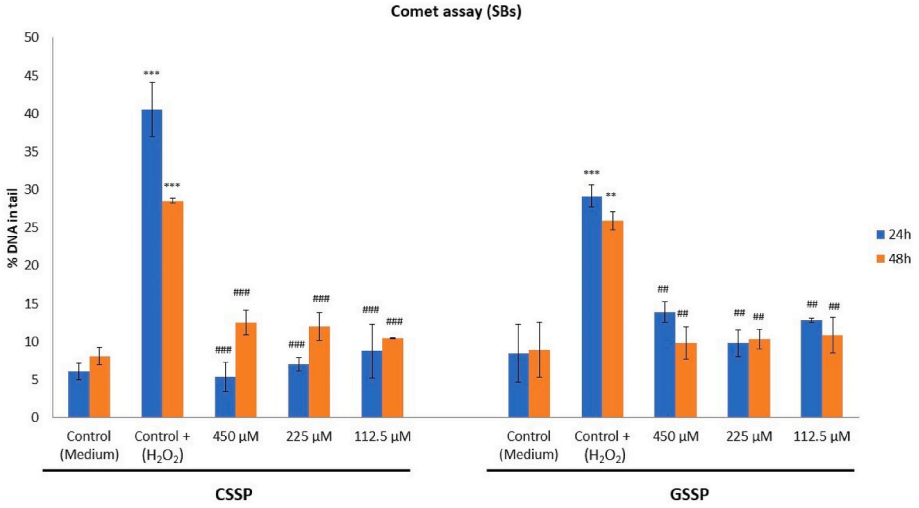

Fig. 2. DNA determined in Caco-2 cells after 24 and 48 h of exposure to CSSP (450–112.5 μM) and GSSP (450–112.5 μM) expressed as the formation of strand breaks (SBs) (% DNA in tail). Positive control was 100 μM H_2O_2. All values are expressed as mean ± SD. The significance levels observed are ***p < 0.001 or **p < 0.01 in comparison to the negative control group values (cellular medium) and ##p < 0.01 or ###p < 0.001 in comparison to the positive control group values.

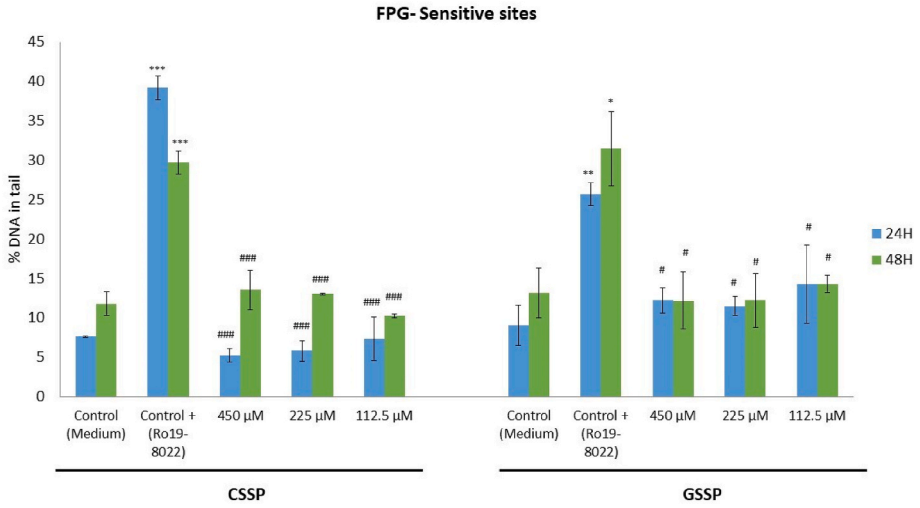

Fig. 3. Oxidative DNA determined in Caco-2 cells after 24 and 48 h of exposure to CSSP (450–112.5 µM) and GSSP (450–112.5 µM) expressed as FPG-sensitive sites (% DNA in tail). Positive control was 2.5 µM Ro19-8022. All values are expressed as mean ± SD. The significance levels observed are ***p < 0.001, **p < 0.01 or *p < 0.05 in comparison to the negative control group values (cellular medium) and #p < 0.05 or ###p < 0.001 in comparison to the positive control group values.

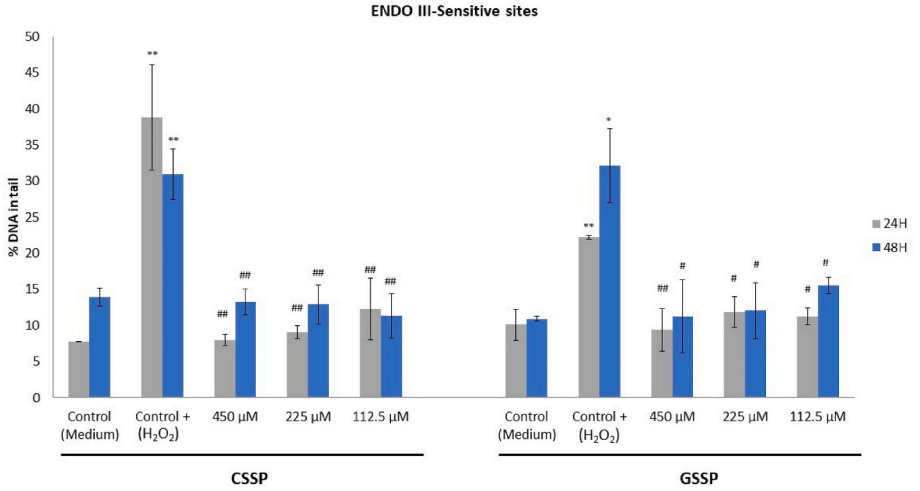

Fig. 4. Oxidative DNA determined in Caco-2 cells after 24 and 48 h of exposure to CSSP (450–112.5 µM) and GSSP (450–112.5 µM) expressed as Endo III-sensitive sites (% tail DNA). Positive control was 100 µM H_2O_2. All values are expressed as mean ± SD. The significance levels observed are **p < 0.01 or *p < 0.05 in comparison to the negative control values (cellular medium) and #p < 0.05 or ##p < 0.01 in comparison to the positive control group values.

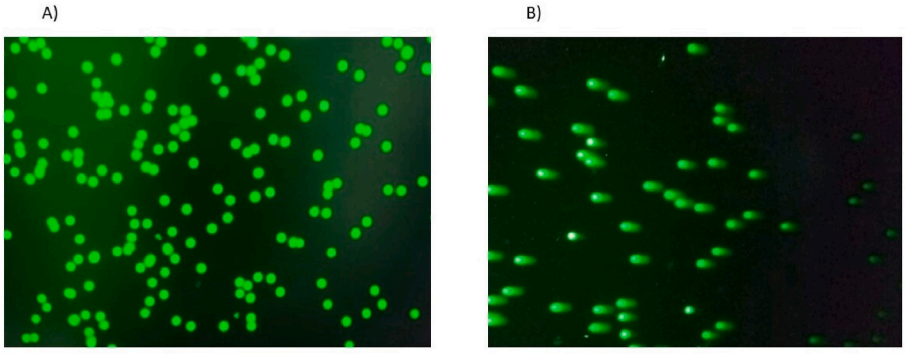

Fig. 5. Fluorescence microscopy (FITC) image of A) nucleus of negative control and B) nucleus of positive control showing DNA in tails.

Investigation; Project administration; Supervision; Roles/Writing - original draft; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Abad, P., Arroyo-manzanares, N., & Ariza, J. J. (2021). Productivity and intestinal microbiota of laying hens. *Animals, 11*, 1–14. https://doi.org/10.3390/ani11010041

Abad, P., Arroyo-Manzanares, N., & García-Campaña, A. M. (2016). A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate, a new additive for animal feed. *Analytical Methods, 8*, 3730–3739. https://doi.org/10.1039/c6ay00219f

Baltrop, J. A., Owen, T. C., Cory, A. H., & Cory, J. G. (1991). 5-((3-Carboxyphenyl)-3-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)) tetrazolium, inner salt (MTS) and related analogs of 2-(4,5-dimethylthiazolyl)-2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell-viability indicators. *Bioorganic & Medicinal Chemistry Letters, 1*, 611.

Borkowska, A., Sielicka-Dudzin, A., Herman-Antosiewicz, A., Wozniak, M., Fedeli, D., Falcioni, G., & Antosiewicz, J. (2012). Diallyl trisulfide-induced prostate cancer cell death is associated with Akt/PKB dephosphorylation mediated by P-p66shc. *European Journal of Nutrition, 51*, 817–825. https://doi.org/10.1007/s00394-011-0260-x

Bradford, M. (1976). A rapid sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cascajosa-Lira, A., Andreo-Martínez, P., Prieto, A. I., Baños, A., Guillamón, E., Jos, Á., & Cameán, A. M. (2022a). In vitro toxicity studies of bioactive organosulfur compounds from allium spp. with potential application in the agri-food industry: A review. *Foods, 11*, 2620. https://doi.org/10.3390/foods11172620

Cascajosa-Lira, A., Pichardo, S., Baños, A., Guillamón, E., Molina-Hernández, V., Moyano, R., Jos, Á., & Cameán, A. M. (2022b). Acute and subchronic 90-days toxicity assessment of propyl-propane-thiosulfinate (PTS) in rats. Food and. *Chemical Toxicology, 161*, Article 112827. https://doi.org/10.1016/j.fct.2022.112827

Cascajosa-Lira, A., Prieto Ortega, A. I., Guzmán-Guillén, R., Cătunescu, G. M., de la Torre, J. M., Guillamón, E., Jos, Á., & Cameán Fernández, A. M. (2021a). Simultaneous determination of *Allium* compounds (Propyl propane thiosulfonate and thiosulfinate) in animal feed using UPLC-MS/MS. *Food and Chemical Toxicology, 157*, Article 112619. https://doi.org/10.1016/j.fct.2021.112619

Cascajosa-Lira, A., Prieto, A. I., Baños, A., Guillamón, E., Moyano, R., Jos, A., & Cameán, A. M. (2020). Safety assessment of propyl-propane-thiosulfonate (PTSO): 90-days oral subchronic toxicity study in rats. *Food and Chemical Toxicology, 144*. https://doi.org/10.1016/j.fct.2020.111612

Cascajosa-Lira, A., Prieto, A. I., Puerto, M., Baños, A., Valdivia, E., Jos, A., & Cameán, A. M. (2020b). Mutagenicity and genotoxicity assessment of a new biopreservative product rich in Enterocin AS-48. *Food and Chemical Toxicology, 146*. https://doi.org/10.1016/j.fct.2020.111846

Cascajosa-Lira, A., Puerto, M., Prieto, A. I., Pichardo, S., Jiménez, L. D. Q., Baños, A., Guillamón, E., Moyano, R., Molina-Hernández, V., Jos, Á., & Cameán, A. M. (2021b). Genotoxicity evaluation of propyl-propane-thiosulfinate (Pts) from *allium* genus essential oils by a combination of micronucleus and comet assays in rats. *Foods, 10*, 989. https://doi.org/10.3390/foods10050989

Catanzaro, E., Canistro, D., Pellicioni, V., Vivarelli, F., & Fimognari, C. (2022). Anticancer potential of allicin: A review. *Phamacology Research, 117*, Article 106118. https://doi.org/10.1016/j.phrs.2022.106118

Collins, A. R., Mitchell, D. L., Zunino, A., de Wit, J., & Busch, D. (1997). UV-sensitive rodent mutant cell lines of complementation groups 6 and 8 differ phenotypically from their human counterparts. *Environmental and Molecular Mutagenesis, 29*, 152–160. https://doi.org/10.1002/(SICI)1098-2280(1997)29:2<152::AID-EM6>3.0.CO;2-G

Cozzolino, R., Malorni, L., Martignetti, A., Picariello, G., Siano, F., Forte, G., & de Giulio, B. (2021). Comparative analysis of volatile profiles and phenolic compounds of Four Southern Italian onion (*Allium cepa* L.) Landraces. *Journal of Food Composition and Analysis, 101*, Article 103990. https://doi.org/10.1016/j.jfca.2021.103990

EFSA CEF Panel. (2016). (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids). Scientific opinion on recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials. *EFSA Journal, 14*, 4357. https://doi.org/10.1016/j.mrgentox.2016.03.001, 10.2903/j.efsa.2016.4357, 800–801, 1–11.

EFSA Panel on. (2012). Food additives and nutrient sources added to food (ANS); guidance for submission for food additive evaluations [53pp.] *EFSA Journal, 10*(7), 2760. https://doi.org/10.2903/j.efsa.2012.2760. efsa.europa.eu/efsajournal).

EFSA Scientific Committee. (2011). Scientific opinion on genotoxicity testing strategies applicable to food and feed safety as-sessment. *EFSA Journal, 9*, 2379.

Farhat, Z., Hershberger, P. A., Freudenheim, J. L., Mammen, M. J., Blair, R. H., Aga, D. S., & Mu, L. (2021). Types of garlic and their an-ticancer and antioxidant activity: A review of the epidemiologic and experimental evidence. *European Journal of Nutrition, 60*(7), 3585–3609. https://doi.org/10.1007/s00394-021-02482-7

Fujii, T., Matsutomo, T., & Kodera, Y. (2018). Changes of S-allylmercaptocysteine and γ-glutamyl-S-allylmercaptocysteine contents and their putative production mechanisms in garlic extract during the aging process. *Journal of Agricultural and Food Chemistry, 66*, 10506–10512. https://doi.org/10.1021/acs.jafc.8b02541

Guillamón, E. (2018). Effect of phytochemical compounds of the genus Allium on the immune system and the inflammatory response. *Ars Pharmaceutica, 59*(3), 185–196. https://doi.org/10.30827/ars.v59i3.7479

Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., & Baños, A. (2021). Beneficial effects of organosulfur compounds from *allium cepa* on gut health: A systematic review. *Foods, 10*. https://doi.org/10.3390/foods10081680

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M. H., & le Bon, A. M. (2000). Liver subcellular fractions from rats treated by organosulfur compounds from *Allium* modulate mutagen activation. *Mutation Research/Genetic Toxicology and Environmental, 466*, 17–26. https://doi.org/10.1016/S1383-5718(99)00234-X

Hsu, C. C., Lin, C. C., Liao, T. S., & Yin, M. C. (2006). Protective effect of s-allyl cysteine and s-propyl cysteine on acetaminophen induced hepatotoxicity in mice. *Food and Chemical Toxicology, 44*, 393–397. https://doi.org/10.1016/j.fct.2005.08.012

Hsu, C. C., Yen, H. F., Yin, M. C., & Tsai, C. M. (2004). Five cysteinecontaining compounds delay diabetic deterioration in Balb/cA mice. *Journal of Nutrition, 134*, 3245–3249. https://doi.org/10.1093/jn/134.12.3245

Imai, T., Kosuge, Y., Endo-Umeda, K., Miyagishi, H., Ishige, K., Makishima, M., & Ito, Y. (2014). Protective effect of S-allyl-l-cysteine against endoplasmic reticulum stress-induced neuronal death is mediated by inhibition of calpain. *Amino Acids, 46*(2), 385–393. https://doi.org/10.1007/s00726-013-1628-4

Kosuge, Y., Koen, Y., Ishige, K., Minami, K., Urasawa, H., Saito, H., & Ito, Y. (2003). S-allyl-l-cysteine selectively protects cultured rat hippocampal neurons from amyloid β-protein- and tunicamycin-induced neuronal death. *Neuroscience, 122*(4), 0–895. https://doi.org/10.1016/j.neuroscience.2003.08.026

Krishnan, V., Loganathan, C., & Thayumanavan, P. (2019). Green synthesized selenium nanoparticle as carrier and potent deliver-ing agent of allyl glutathione: Anticancer effect against hepatocarcinoma cell line (HepG2) through induction of cell cy-cle arrest and apoptosis. *Journal of Drug Delivery Science and Technology, 53*, Article 101207. https://doi.org/10.1016/j.jddst.2019.101207

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Moreno, F. J., Baños, A., Nuñez, C., Guillamón, E., & Cameán, A. M. (2015a). Acute toxicological studies of the main organosulfur compound derived from *Allium* sp. intended to be used in active food packaging. *Food and Chemical Toxicology, 82*, 1–11. https://doi.org/10.1016/j.fct.2015.04.027

Llana-Ruiz-Cabello, M., Maisanaba, S., Gutiérrez-Praena, D., Prieto, A. I., Pichardo, S., Jos, Á., Moreno, F. J., & Cameán, A. M. (2015b). Cytotoxic and mutagenic *in vitro* assessment of two organosulfur compounds derived from onion to be used in the food industry. *Food Chemistry, 166*, 423–431. https://doi.org/10.1016/j.foodchem.2014.06.058

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A. I., Pichardo, S., Jos, Á., & Cameán, A. M. (2014). Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames *Salmonella* test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. *Food and Chemical Toxicology, 72*, 122–128. https://doi.org/10.1016/j.fct.2014.07.013

Maisanaba, S., Prieto, A. I., Puerto, M., Gutiérrez-Praena, D., Demir, E., Marcos, R., & Cameán, A. M. (2015). *In vitro* genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays. *Mutation Research/Genetic Toxicology and Environmental, 784–785*, 37–44. https://doi.org/10.1016/j.mrgentox.2015.05.005

Marimuthu, M. K., & Ramasamy, T. (2020). Protective effect of diallyl disulfide against cerulein induced acute pancreatitis and as-sociated lung injury in mice. *International Immunopharmacology, 80*, Article 106136. https://doi.org/10.1016/j.intimp.2019.106136

Martínez-Fernández, G., Abecia, L., Martín-García, A. I., Ramos-Morales, E., Denman, S. E., Newbold, C. J., Molina-Alcaide, E., & Yáñez-Ruiz, D. R. (2015). Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. *FEMS Microbiology Ecology, 91*, 1–11. https://doi.org/10.1093/femsec/fiv079

Medrano-Padial, C., Prieto, A. I., Puerto, M., & Pichardo, S. (2021). *In vitro* assessment of the mutagenic and genotoxic potential of a pure stilbene extract. *Food and Chemical Toxicology, 150*, Article 112065. https://doi.org/10.1016/j.fct.2021.112065

Mellado-García, P., Maisanaba, S., Puerto, M., Llana-Ruiz-Cabello, M., Prieto, A. I., Marcos, R., Pichardo, S., & Cameán, A. M. (2015). Genotoxicity assessment of propyl thiosulfinate oxide, an organosulfur compound from *Allium* extract, intended to food active packaging. *Food and Chemical Toxicology, 86*, 365–373. https://doi.org/10.1016/j.fct.2015.11.011

Mellado-García, P., Maisanaba, S., Puerto, M., Prieto, A. I., Marcos, R., Pichardo, S., & Cameán, A. M. (2017). *In vitro* toxicological assessment of an organosulfur compound from *Allium* extract: Cytotoxicity, mutagenicity and genotoxicity studies. *Food and Chemical Toxicology, 99*, 231–240. https://doi.org/10.1016/j.fct.2016.12.007

Mellado-García, P., Puerto, M., Prieto, A. I., Pichardo, S., Martín-Cameán, A., Moyano, R., Blanco, A., & Cameán, A. M. (2016). Genotoxicity of a thiosulfonate compound derived from *allium* sp. intended to be used in active food packaging: *In vivo* comet assay and micronucleus test. *Mutation Research/Genetic Toxicology and Environmental, 800–801*, 1–11. https://doi.org/10.1016/j.mrgentox.2016.03.001

Mong, M., & Yin, M. (2012). Nuclear factor κb-dependent anti-inflammatory effects of S -allyl cysteine and S-propyl cysteine in kidney of diabetic mice. *Journal of Agricultural and Food Chemistry, 60*(12), 3158–3165. https://doi.org/10.1021/jf3002685

Mylona, K., Garcia-Cela, E., Sulyok, M., Medina, A., & Magan, N. (2019). Influence of two garlic-derived compounds, propyl propane thiosulfonate (PTS) and propyl propane thiosulfinate (PTSO), on growth and mycotoxin production by Fusarium species. *Vitro and in Stored Cereals. Toxins, 11*(9), 495. https://doi.org/10.3390/toxins11090495

OECD. (2016). Test No. 487. In *Vitro mammalian cell micronucleus test, OECD guidelines for the testing of chemicals, section 4*. Paris: OECD Publishing. https://doi.org/10.1787/9789264264861-en.

OECD. (2020). *Test No. 471: Bacterial reverse mutation test, OECD guidelines for the testing of chemicals, section* (Vol. 4). Paris: OECD Publishing. https://doi.org/10.1787/9789264071247-en

Poojary, M. M., Putnik, P., Bursać Kovačević, D., Barba, F. J., Lorenzo, J. M., Dias, D. A., & Shpigelman, A. (2017). Stability and extraction of bioactive sulfur compounds from *Allium* genus processed by traditional and innovative technologies. *Journal of Food Composition and Analysis, 61*, 28–39. https://doi.org/10.1016/j.jfca.2017.04.007

Puerto, M., Prieto, A. I., Pichardo, S., Moreno, I. M., Jos, A., Moyano, R., & Camean, A. M. (2009). Effects of dietary N-acetylcysteine on the oxidative stress induced in tilapia (*oreochromis niloticus*) exposed to a microcystin-producing cyanobacterial water bloom. *Environmental Toxicology and Chemistry, 28*, 679–1686. https://doi.org/10.1897/08-520.1

Putnik, P., Gabrić, D., Roohinejad, S., Barba, F. J., Granato, D., Mallikarjunan, K., Lorenzo, J. M., & Bursać Kovačević, D. (2019). An overview of organosulfur compounds from *Allium* spp.: From processing and preservation to evaluation of their bioavailability, antimicrobial, and anti-inflammatory properties. *Food Chemistry, 276*, 680–691. https://doi.org/10.1016/j.foodchem.2018.10.068

Rabelo-Ruiz, M., Ariza-Romero, J. J., Zurita-González, M. J., Martín-Platero, A. M., Baños, A., Maqueda, M., Valdivia, E., Martínez-Bueno, M., & Peralta-Sánchez, J. M. (2021). Allium-based phytobiotic enhances egg production in laying hens through microbial composition changes in ileum and cecum. *Animals, 11*, 1–15. https://doi.org/10.3390/ani11020448

Rodrigues-Barreto, M., Aleixo, N. A., Silvestre, R. B., Fregonezi, N. F., Barud, H. da S., Dias, D. dos S., Ribeiro, C. A., & Resende, F. A. (2019). Genotoxicological safety assessment of puree-only edible films from onion bulb (*Allium cepa* L.) for use in food packaging-related applications. *Journal of Food Science, 85*, 201–208. https://doi.org/10.1111/1750-3841.14977

Roy, N., Nazeem, P. A., Babu, T. D., Abida, P. S., Narayanankutty, A., Valsalan, R., Valsala, P. A., & Raghavamenon, A. C. (2017). EGFR gene regulation in colorectal cancer cells by garlic phytocompounds with special emphasis on S-Allyl-L-Cysteine Sulfoxide. *Interdisciplinary Sciences: Computational Life Sciences, 10*, 686–693. https://doi.org/10.1007/s12539-017-0227-6

Sielicka-Dudzin, A., Borkowska, A., Herman-Antosiewicz, A., Wozniak, M., Jozwik, A., Fedeli, D., & Antosiewicz, J. (2012). Impact of JNK1, JNK2, and ligase Itch on reactive oxygen species formation and survival of prostate cancer cells treated with diallyl trisulfide. *European Journal of Nutrition, 51*, 573–581. https://doi.org/10.1007/s00394-011-0241-0

Thayumanavan, P., Loganathan, C., Iruthayaraj, A., Poomani, K., & Nallaiyan. (2017). S-Allyl-glutathione, a synthetic analogue of glutathione protected liver against carbon tetrachloride toxicity: Focus towards anti-oxidative efficiency. *Environmental Toxicology and Pharmacology, 58*, 21–28. https://doi.org/10.1016/j.etap.2017.12.006

Tsai, S. J., Chiu, C. P., Yang, H. T., & Yin, M. C. (2011). s-Allyl cysteine, sethyl cysteine, and s-propyl cysteine alleviate β-amyloid, glycative, and oxidative injury in brain of mice treated by D galactose. *Journal of Agricultural and Food Chemistry, 59*, 6319–6326. https://doi.org/10.1021/jf201160a

Wu, P. P., Chung, H. W., Liu, K. C., Wu, R. S. C., Yang, J. S., Tang, N. Y., Lo, C., Hsia, T. E. C., Yu, C. C., Chueh, F. U. S., Lin, S. S., & Chung, J. G. (2011). Diallyl sulfide induces cell cycle arrest and apoptosis in HeLa human cervical cancer cells through the p53, caspase- and mitochondria-dependent pathways. *International Journal of Oncology, 38*, 1605–1613. https://doi.org/10.3892/ijo.2011.973

Xiao, J., Xing, F., Liu, Y., Lv, Y., Wang, X., Ling, M.-T., Gao, H., Ouyang, S., Yang, Zhu, J., Xia, Y., So, K.-F., & Tipoe, G. L. (2018). Garlic-derived compound S-allylmercaptocysteine inhibits hepatocarcinogenesis through targeting LRP6/Wnt pathway. *Acta Pharmaceutica Sinica B, 8*(4), 575–586. https://doi.org/10.1016/j.apsb.2017.10.003

Yao, H., Peng, Y., & Zheng, J. (2016). Identification of glutathione and related cysteine conjugates derived from reactive metabolites of methyleugenol in rats. *Chemistry & Biology, 253*, 143–152. https://doi.org/10.1016/j.cbi.2016.05.006

Zhang, G., Li, B., Lee, C.-H., & Parkin, K. L. (2010). Cysteine and glutathione mixed disulfide conjugates of thiosulfinates: Chemical synthesis and biological activities. *Journal of Agricultural and Food Chemistry, 58*, 1564–1571. https://doi.org/10.1021/jf9029354

Zhang, Y., Li, H.-Y., Zhang, Z.-H., Bian, H.-L., & Lin, G. (2014). Garlic-derived compound S-allylmercaptocysteine inhibits cell growth and induces apoptosis via the JNK and p38 pathways in human colorectal carcinoma cells. *Oncology Letters, 8*, 2591–2596. https://doi.org/10.3892/ol.2014.2579

Zhong, B. Z., Yao, H., Peng, Y., Zheng, J., Depree, G. J., & Siegel, P. D. (2001). Differentiation of the mechanism of micronuclei induced by cysteine and glutathione conjugates of methylenedi-p-phenyl diisocyanate from that of 4,4′-methylenedianiline. *Chemistry & Biology, 497*, 143–152. https://doi.org/10.1016/j.cbi.2016.05.006